

Dominant Clonotypes in the Repertoire of Peripheral CD4⁺ T Cells in Rheumatoid Arthritis

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Abstract

Clonal expansion of T cell specificities in the synovial fluid of patients has been taken as evidence for a local stimulation of T cells. By studying the T cell receptor (TCR) repertoire of CD4⁺ T cells in the synovial and peripheral blood compartments of patients with early rheumatoid arthritis (RA), we have identified clonally expanded CD4⁺ populations. Expanded clonotypes were present in the peripheral blood and the synovial fluid but were not preferentially accumulated in the joint. Dominant single clonotypes could not be isolated from CD4⁺ cells of HLA-DRB1*04⁺ normal individuals. Clonal expansion involved several distinct clonotypes with a preference for V β 3⁺, V β 14⁺, and V β 17⁺CD4⁺ T cells. A fraction of clonally related T cells expressed IL-2 receptors, indicating recent activation. The frequencies of clonally expanded V β 17⁺CD4⁺ T cells fluctuated widely over a period of one year. Independent variations in the frequencies of two distinct clonotypes in the same patient indicated that different mechanisms, and not stimulation by a single arthritogenic antigen, were involved in clonal proliferation. These data support the concept that RA patients have a grossly imbalanced TCR repertoire. Clonal expansion may result from intrinsic defects in T cell generation and regulation. The dominance of expanded clonotypes in the periphery emphasizes the systemic nature of RA and suggests that T cell proliferation occurs outside of the joint. (*J. Clin. Invest.* 1994. 94:2068–2076.) Key words: T cell receptor • V β gene segment • immunoregulation • T cell diversity

Introduction

RA is a chronic inflammatory disease of unknown etiology (1). The presence of CD4⁺ T cells in the synovial lesions has supported the hypothesis that T helper cells are important in inducing and sustaining the synovial inflammation (2–4). Genetic studies identifying a shared sequence polymorphism encoded by the third hypervariable region (HVR)¹ of the HLA-DRB1 gene have supported the theory that pathomechanisms in RA are related to the ternary complex formed by HLA mole-

cules, antigenic peptides, and interacting T cell receptor (TCR) molecules (5–9). In the current paradigm, disease associated HLA-DR molecules function by selecting and presenting arthritogenic antigens to tissue infiltrating T cells. So far, no disease-inducing antigen has been identified. Recent efforts have focused on analyzing the molecular diversity of the T cell population accumulated in the synovia (10–16). Some, but not all reports have indicated that the diversity of synovial T cells is restricted, raising the possibility that selected T cell specificities encounter antigen in the joint and proliferate. The model of selective representation of some T cell specificities in the synovia, however, has been challenged by a lack of consensus in different reports (17). A further challenge to the model of a synovia specific T cell response to an arthritogenic antigen comes from the clinical experience that RA is a systemic disease. Morbidity and mortality of the disease are associated with progression of the disease beyond the joint.

Several recent observations are difficult to reconcile with the model that the HLA-DR association of RA solely reflects the selective peptide binding of HLA-DR molecules in the induction or effector phase of the pathogenetic immune response. We have recently described that HLA-DRB1 alleles influence severity of the disease rather than susceptibility. In particular, a gene dosage effect of HLA-DRB1 alleles is functional in RA (8, 18). Patients combining two disease-associated HLA-DRB1 alleles have not only more severe joint destruction but also a significant risk of developing extra-articular disease. A unique role exists for homozygosity of the HLA-DRB1*0401 allele, a genotype accumulated in the patient cohort with a very high frequency of rheumatoid vasculitis. We have therefore proposed the alternative model that the contribution of disease linked HLA-DR molecules relates to their role in forming the TCR repertoire (8, 19).

In studies comparing the synovial and peripheral TCR repertoires in RA patients, we have now made the observation that RA patients carry clonally expanded populations of CD4⁺ T cells. Expanded clonotypes were highly frequent in the peripheral blood. While they were present in synovial fluid and blood, they were not specifically enriched in the joint. Oligoclonality preferentially involved V β 3⁺, V β 14⁺, and V β 17⁺ T cells. Different clonotypes fluctuated independently in their frequencies, suggesting that recognition of a single antigen is not the driving force for clonal expansion. These data demonstrate an imbalance in the TCR repertoire of CD4⁺ helper cells in RA patients which may relate to a defect in generating or controlling diversity of CD4⁺ T cells.

Methods

Patients. Five patients with RA and a disease duration of less than 6 mo after the first onset of symptoms were studied. All patients were

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1. Abbreviations used in this paper: CDR, complementarity determining region; HVR, hypervariable region; TCR, T cell receptor.

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followed for at least 12 mo to confirm the diagnosis of RA according to the ACR criteria (20). Control individuals were selected to match the HLA-DRB1 alleles of the patient population. They did not have a personal or family history of RA or any other chronic inflammatory disease.

Patients and controls were characterized for their HLA-DRB1 alleles by allele-specific amplification and subsequent oligonucleotide hybridization as recently described (8, 21). In brief, DNA was extracted from PBMC and amplified with primer sets specific for the sequence polymorphism in the HVR1 of the HLA-DRB1 alleles. Amplified sequences were subsequently hybridized with oligonucleotides specific for polymorphism of the HVR2 and HVR3. The sequences of the oligonucleotides used have been recently described. The HLA-DRB1 alleles of patients RA-1 to RA-5 and the control individuals NP-1 to NP-8 were as follows: RA-1, HLA-DRB1*0404/0404; RA-2, HLA-DRB1*0101/0404; RA-3, HLA-DRB1*0401/13; RA-4, HLA-DRB1*0101/13; RA-5, HLA-DRB1*0101/0404; NP-1, HLA-DRB1*0101/0401; NP-2, HLA-DRB1*0101/0401; NP-3, HLA-DRB1*0401/1402; NP-4, HLA-DRB1*0101/0401; NP-5, HLA-DRB1*04/07; NP-6, HLA-DRB1*0401/13; NP-7, HLA-DRB1*15 or 16/0404; NP-8, HLA-DRB1*0101/13.

T cell cloning. Synovial fluid cells were directly cloned in the presence of rIL-2 (20 U/ml; Cetus Corp., Emeryville, CA) and 5×10^4 /well irradiated HLA-DRB1 matched filler cells at a concentration of four cells/well. PBMC were activated in vitro with immobilized anti-CD3 for 12 h at a concentration of 1×10^6 /ml and then cloned in IL-2 containing medium at 0.5 cell/well. Peripheral T cell clones from patient RA-1 were obtained from three independent experiments to ascertain that the identification of identical clonotypes did not represent an in vitro culture artifact. Established T cell clones were screened for the expression of the CD4 and CD8 markers by FACS analysis. Only cultures which were negative for CD8 cells were further analyzed. T cell clones (58, 70, and 71 clones, respectively) were established from individuals NP-1, NP-2, and NP-3, using the identical procedure described for the RA patients. PBMC from individuals NP-4 and NP-5 were stained with mAb to CD4 (Becton Dickinson, Mountain View, CA) and V β 17 (Amac Inc., Westbrook, ME) and sorted on a FACS Vantage. A minimum of 10,000 V β 17⁺ CD4⁺ T cells was obtained and activated with immobilized anti-CD3 for 12 h in the presence of syngeneic irradiated filler cells. T cells were then cloned as described above.

V β gene segment analysis of T cell clones. Total RNA was extracted from CD4⁺ T cell clones by guanidinium thiocyanate phenol chloroform extraction using a commercially available kit (RNA Stat; Tel Test, Friendswood, TX). V β gene segment usage was determined by reverse transcription PCR with V β specific primers and a C β specific primer as recently described (22, 23). Amplified products were separated on 2% agarose gels and identified with ethidium bromide.

TCR sequence analysis of T cell clones. cDNA from selected T cell clones was amplified with a C β primer and the appropriate V β primer attached to a T7 promoter. The amplified product was transcribed using a T7 RNA polymerase and directly sequenced by reverse transcriptase mediated dideoxysequencing as recently described (24, 25).

CDR3 length analysis of V β /J β transcripts. CD4⁺ IL-2R⁻ from PBMC of patients and normal controls were purified by cell sorting on a FACS Vantage. Total RNA was extracted, reverse transcribed, and amplified with primers specific for V β 3, V β 5, V β 8, V β 14, and V β 17 and a C β primer. 0.5 μ l of the amplified product was then reamplified by using an end-labeled J β specific primer and the appropriate V β primer. The following J β primers were used: 1.1, AACTGTGAGTCTGGTGCCTTGT; 1.2, ACCTGGTCCCCGAACCGAAGG; 1.5, GATGGAGATCGAGTCCCATCA; 2.1, CCTCTAGCACGGTGAGCCGT; 2.3, TGCCTGGGCCAAAATACT; 2.5, ACCAGGAGCCCGTGCCT; and 2.7, ACCGTGAGCCTGGTGCCC. The amplified products were separated on a 5% acrylamide sequencing gel. For the majority of samples, the analysis showed 8–12 bands for one particular V-J combination with a Gaussian distribution of the band intensities. Samples which deviated from the Gaussian distribution or had a dominant

band were identified and analyzed by direct sequencing using the appropriate J β specific primer as a sequence initiation primer. The sensitivity of the direct sequencing approach was analyzed by serially diluting T cell clones with known TCR β chain sequences with PBMC samples with known V β frequencies as determined by FACS[®] analysis. Total RNA was extracted from the different dilutions, reverse transcribed, amplified with the appropriate V β specific primer set, and then directly sequenced using an internal C β specific primer as sequence initiation primer. These experiments demonstrated that the frequency of one particular clonotype had to be larger than 25% of all T cells sharing the particular V β element to give an unequivocal sequence (data not shown).

Dot blot assay with CDR3 and J β specific primers. PBMC and synovial fluid cells were activated with immobilized anti-CD3 and cultured with 20 U/ml rIL-2. Cells were depleted of CD8⁺ cells after 14 d by incubation with 40 μ g/10⁶ cells anti-CD8 (OKT8, CRL 8021; American Type Culture Collection, Rockville, MD) and subsequent incubation with magnetic beads coupled with anti-mouse Ig (Advanced Magnetics, Cambridge, MA). CD8⁺ cells were removed, total RNA was extracted from the CD8 depleted population, and cDNA was synthesized. To enrich for cells expressing a functional IL-2 receptor, 1×10^6 /ml PBMC and synovial cells, respectively, were cultured in medium containing low doses of IL-2 (20 U/ml) without prior in vitro activation. Proliferating cells were harvested after 14 d and depleted of CD8⁺ cells as described above. Oligonucleotides were designed for J β 1.2 (CCTTCGGTTCGGGGACCAGGT), J β 2.2 (GAAGGCTCTAGGCTGACCGTA), J β 2.5 (CGGGCCAGGCACGCGGCTCTG), and J β 2.7 (CCGGGCACCAGGCTCACGGTC) and for the CDR3 sequence of the expanded clonotype in patient RA-1 (RA-1-17A, GGACAACCTA-TGGCTACACCTTC). All oligonucleotides were conjugated with biotin. cDNA was amplified with the V β 17 specific primer set, and the amplified templates were adjusted. Serial dilutions were vacuum blotted onto supported nitrocellulose membranes, prehybridized, and hybridized with the biotinylated probes and biotinylated internal C β probe (AGAGAGCGACCTCGGGTGGGA), respectively, at 55°C. Blots were washed for 10 min at 42°C and 10 min at 55°C in 2 \times SSC 0.1 SDS, blocked with BSA blocking buffer, and developed with streptavidin-alkaline phosphatase. Signals were scanned using the AMBIS Imaging System (San Diego, CA). CDR3 specific and J β specific signals were normalized to the C β specific signal. Serial dilutions of amplified templates from T cell clones expressing the CDR3 sequence or the appropriate J β element were used as internal standards to determine the frequencies of these sequences in the peripheral and synovial CD4⁺ T cell compartments.

Results

Clonally expanded CD4⁺ T cells in the synovial fluid and peripheral blood of RA patients. To address the question whether the induction of an immune response in the synovia of RA patients involves a limited number of T cell specificities, we have analyzed the diversity of synovial fluid T cells in patients with early RA. Samples of synovial fluid were obtained in two patients presenting with < 3 mo of symptoms. Patients were followed for at least one year and were selected for TCR studies if they developed unequivocal RA and expressed at least one disease associated HLA-DRB1 allele. To select for in vivo activated T lymphocytes, synovial T cells were directly cloned on HLA-DRB1 matched irradiated filler cells in the presence of 20 U/ml rIL-2. CD4⁺ T cell clones were identified, and a reverse transcription-PCR with V β specific primer sets was used to identify the TCR V β element expressed by each T cell clone. V β analysis of established T cell clones demonstrated that T cells in the synovial fluid were polyclonal (Fig. 1). To investigate whether, within the heterogeneous T cell populations, some

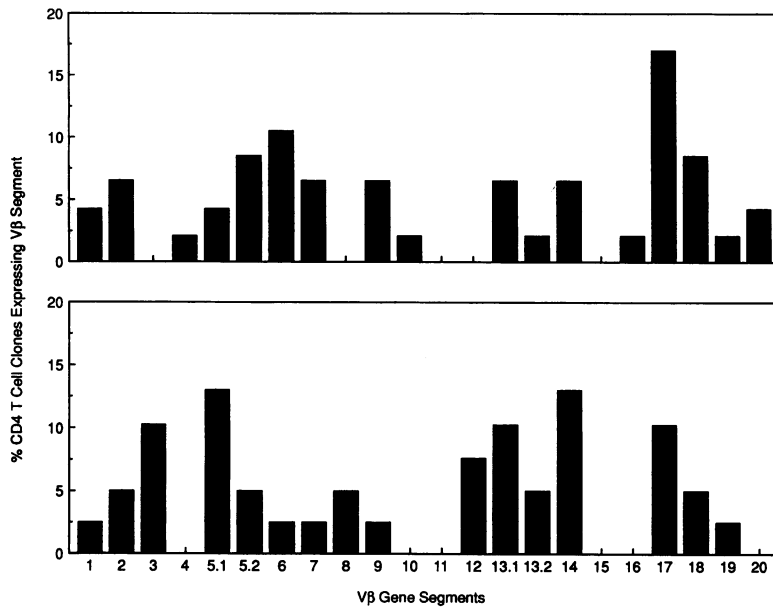


Figure 1. V β gene segment usage of synovial CD4⁺ T cell clones derived from two RA patients with early disease. Synovial fluid mononuclear cells of two patients with early RA were directly cloned without prior in vitro activation. CD4⁺ T cell clones were characterized for their V β gene segment usage by PCR with V β specific primer sets. Results are given for 47 synovial T cell clones from patient RA-1 and 39 from patient RA-2.

specificities were clonally expanded, we sequenced TCR β chains of V β identical clones. Sequence analysis revealed that few T cell specificities were clonally expanded in the synovial fluid.

In patient RA-1 we found four different sequences within a group of seven V β 17⁺ T cell clones. One clonal specificity was represented three times, another one two times. Out of three V β 14⁺ T cell clones, two utilized identical TCR β chains. Evidence for oligoclonal expansion was not found for any other V β gene segment analyzed, suggesting that this phenomenon may be restricted to V β 14⁺ and V β 17⁺ T cell clones. From the second patient we obtained three V β 14⁺ T cell clones, two of which were identical.

To address the question whether the clonal expansion was specific for the synovial fluid or whether the expanded clonotypes were also found in the peripheral blood, CD4⁺ T cell clones were established from peripheral blood after polyclonal activation and TCR β genes from V β 14⁺ and V β 17⁺ T cell clones were sequenced. The sequence data of synovial and peripheral T cell clones are summarized in Tables I and II. Of the four clonotypes found to be expanded in the synovial fluid, two were found to be also dominant in the peripheral blood. In particular, the repertoire of V β 17⁺ T cells in patient RA-1

was highly restricted and was clearly dominated by one clonal specificity which was found in 24 of 30 V β 17⁺ peripheral T cell clones derived from three independent cloning experiments at two different time points (months 2 and 7 after disease onset) and in two out of seven synovial T cell clones. A second expanded clonotype, RA-1-17C, was identified only in the peripheral blood at month 7. Also, the V β 14⁺ T cell clonotype, which had been isolated from the synovial fluid, was present in the peripheral blood. A very similar picture emerged for patient RA-2. In 52 CD4⁺ peripheral T cell clones analyzed, we found eight V β 14⁺ T cell clones and four V β 17⁺ clones. Sequencing TCR genes from these T cell clones demonstrated that seven V β 14⁺ T cell clones and three V β 17⁺ T cell clones were identical. The peripheral V β 14 T cell clone, which was found in multiple copies, had the same sequence as the two identical synovial T cell clones. A total of 20 additional T cell clones expressing the V β 2, V β 3, V β 5.1, or V β 13.1 gene segment from this patient were sequenced. All of these T cell clones carried distinct receptor molecules, suggesting that the phenomenon of clonal expansion was restricted to the V β 14⁺ and V β 17⁺ T cell subsets.

Frequency of clonally expanded T cell specificity in peripheral IL-2R⁻ and IL-2R⁺ T cells. To analyze the distribution of

Table I. Clonal Expansion of CD4⁺ T Lymphocytes in Peripheral Blood and Synovial Fluid of Patients with Early RA

Donor	V β element	TCR β chains sequenced	No. of identical clonotypes	Source of identical clonotypes	Expanded clonotype
RA-1	V β 14	n = 4	n = 2	Synovia	RA1-14A
			n = 2	Synovia and peripheral blood	RA1-14B
	V β 17	n = 37	n = 26	Synovia and peripheral blood	RA1-17A
			n = 3	Synovia	RA1-17B
RA-2	V β 14	n = 12	n = 2	Peripheral blood	RA1-17C
			n = 9	Synovia and peripheral blood	RA2-14A
			n = 3	Peripheral blood	RA2-17A

Table II. Junctional Sequences of Expanded Clonotypes

Expanded clonotype	V β	N-D-N	J β
RA1-14A	C A S S TGT GCC AGC AGT	S G T A S S Y N TCT GGG ACG GCA AGC TCC TAT AAT	S P L H F TCA CCC CTC CAC TTT (1.6)
RA1-14B	C A S S TGT GCC AGC AGT	T T R G T G ACC ACC AGA GGG ACG GGA	Y G Y T F TAT GGC TAC ACC TTC (1.2)
RA1-17A	C A S TGT GCC AGT	R D AGG GAC	N Y G Y T F AAC TAT GGC TAC ACC TTC (1.2)
RA1-17B	C A S S TGT GCC AGT AGT	L G L R T G CTG GGG CTG AGG ACA GGA	E A F F GAA GCT TTC TTT (1.1)
RA1-17C	C A S S TGT GCC AGT AGT	P R G P P S V H CCT CGA GGG CCT CCC TCG GTC CAC	E Q Y F GAG CAG TAC TTC (2.7)
RA2-14A	C A S S TGT GCC AGC AGT	L R E G CTA AGA GAG GGG	E Q F F GAG CAG TTC TTC (2.1)
RA2-17A	C A S S TGT GCC AGT AGT	T S G Q V ACG AGT GGA CAG GTC	T D T Q Y F ACA GAT ACG CAG TAT TTT (2.3)

expanded clonotypes in the peripheral blood compartment, IL-2R⁺ and IL-2R⁻ CD4⁺ peripheral T cells from patient RA-1 were purified by cell sorting. Total RNA was extracted, reverse transcribed, and amplified with a V β 17 specific primer set. The amplified products were directly sequenced by using a J β 1.2 specific primer as sequence initiation primer. These experiments resulted in unequivocal sequences, demonstrating that the major clonotype, RA-1-17A, constituted a major fraction of the peripheral compartment of this patient. To more accurately estimate the frequency of clonally expanded T cell specificities in peripheral blood, a hybridization assay with oligonucleotides complementary to the CDR3 sequence of clone RA-1-17A was established. cDNA samples were amplified with a V β specific primer set, and the amplified product was probed with the CDR3 specific probe. Fig. 2 shows the result of PBMC from patient RA-1 obtained at the first time point, 2 mo after the onset of

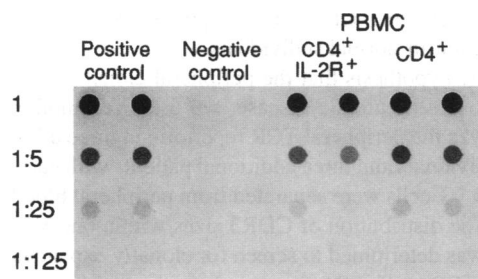


Figure 2. Representation of dominant clonotype RA-1-17A in peripheral blood CD4⁺ T cells and in preactivated CD4⁺ T cells. Total RNA was extracted from CD4⁺ T cells and from IL-2 responsive CD4⁺ T cells from peripheral blood of patient RA-1 2 mo after onset of symptoms. cDNA was amplified with a V β 17 specific primer set. Serial dilutions of the amplified product were hybridized with a biotinylated probe complementary to the CDR3 sequence of the clonotype RA-1-17A (Table II). cDNA from a T cell clone expressing the CDR3 sequence served as positive control, a T cell clone with the same V β 17-J β 1.2 combination but a different junctional sequence as negative control. Signals were scanned by using the AMBIS system. CDR3 sequences were found in peripheral CD4⁺ T cells and in CD4⁺ T cells enriched for IL-2R⁺ cells.

subjective symptoms. Serial dilutions of cDNA from the T cell clone expressing the appropriate CDR3 served as positive controls. The probe was specific for CDR3 specific sequences and did not cross-hybridize on a T cell clone sharing the same V β -J β combination but expressing a different junctional sequence. CDR3 specific sequences were detected in CD4⁺ and CD4⁺ IL-2R⁺ PBMC. Frequencies were determined by parallel hybridization of the amplified product with a C β specific probe (data not shown) and by comparing the signal intensities with serial dilutions of the positive control clone. The clonal specificity was not enriched in the IL-2R⁺ fraction compared to the total CD4 population (24% versus 39% of V β 17 transcripts).

Peripheral CD4⁺ T cells from patient RA-1 were studied over a period of 12 mo. The longitudinal time course of the frequencies of clonal specificity RA-1-17A is given in Fig. 3. This clonotype represented a substantial proportion of CD4⁺ V β 17⁺ T cells which ranged from 30 to 40% in the first 10 mo and increased to over 80% after 15 mo (Fig. 3, upper panel). Studies with mAb specific for V β 17 demonstrated frequencies in the high normal range from 5 to 7% between 2 mo after the disease onset and 10 mo after disease onset. The frequencies increased to 15% V β 17⁺ T cells of peripheral CD4⁺ T cells 15 mo after disease onset, at the time when this clonal specificity had expanded to more than 80% of V β 17⁺ T cells. At any given time point, the clonal specificity was found within the subset of IL-2R⁺ T cells indicating recent activation (Fig. 3, lower panel). No striking correlation between the frequency of the T cell clone within the CD4⁺ subset and the frequency within the CD4⁺ IL-2R⁺ subset was found. In the first 10 mo of disease, the frequency of the clonotype within the IL-2 responsive cells varied between 9 and 34%. 15 mo after disease onset, the clonotype was found only at a frequency of 6% within CD4⁺ IL-2R⁺ cells, while at this time point, the frequency of the T cell clone had increased to over 80% of all CD4⁺ V β 17⁺ T cells.

Distribution of clonally expanded T cells in the peripheral and synovial compartments. The expanded clonotypes were originally isolated from the synovial fluid and subsequently found in high frequencies in the peripheral blood compartment.

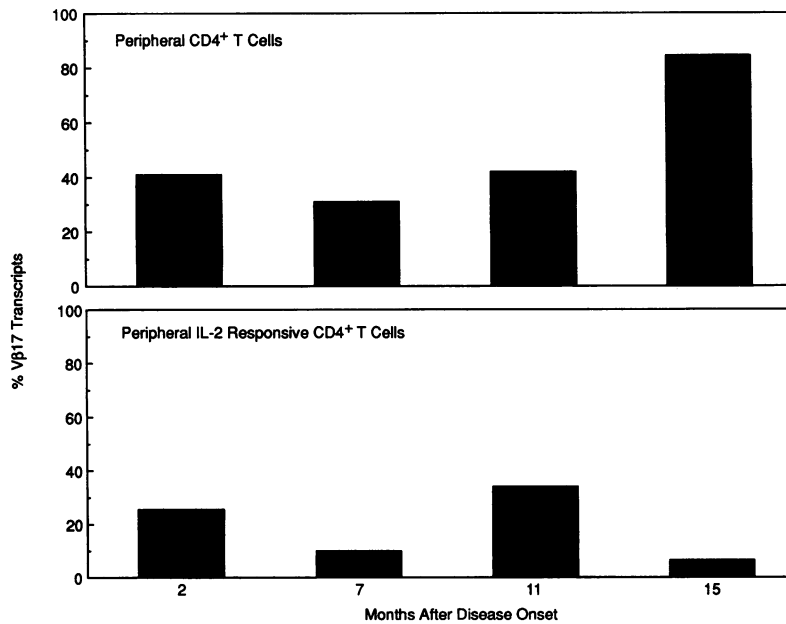


Figure 3. Longitudinal time course of clonotype RA-1-17A transcripts in peripheral blood of patient RA-1. CD4⁺ T cells (*upper panel*) and preactivated IL-2R⁺ CD4⁺ T cells (*lower panel*) were obtained from peripheral blood of patient RA-1 at 2, 7, 11, and 15 mo after disease onset. cDNA was amplified with a Vβ17 specific primer set and the amplified product was probed with a CDR3 specific and a Cβ specific oligonucleotide as described in Fig. 2. Frequencies were calculated by comparing the signal intensities with serial dilutions of a positive control clone.

The current paradigm predicts that the disease relevant immune response in RA occurs in the synovial membrane. To address the question whether the expanded clonotypes were accumulated in the synovia or selectively activated in the synovia, we compared frequencies of CDR3 specific sequences in synovial and peripheral T cells. As summarized in Table III, there was no evidence for an enrichment of the sequence specific for the CDR3 of clone RA-1-17A in the synovial fluid. 7 mo after disease onset of patient RA-1, the clone was fivefold more frequent in the peripheral blood compartment than in the synovial fluid. While the clone was present in the IL-2R⁺ peripheral CD4⁺ compartment, albeit at a lower frequency than in the IL-2R⁻ compartment, preactivated cells of this particular clonotype were almost undetectable in the synovial fluid. 8 mo later, the clonotype represented the vast majority of circulating and synovial Vβ17⁺ T cells. The frequency of the clonotype was significantly lower in the IL-2R⁺ CD4⁺ T cells in both compartments (6% of peripheral CD4⁺ IL-2R⁺ Vβ17⁺ cells vs 15% of synovial IL-2R⁺ CD4⁺ Vβ17⁺ cells).

Clonal expansion of peripheral CD4⁺ T cells is frequently found in patients with early RA. The distribution of the expanded clonotypes in the periphery and in the synovial fluid of patients RA-1 and RA-2 did not support the concept that the joint was the site of clonal expansion. To address the possibility that clonal expansion of peripheral CD4⁺ T cells is a general

phenomenon in normal individuals and that the finding of these expanded clonotypes in the synovial fluid only reflected the homing pattern of expanded clonotypes, we analyzed normal healthy donors expressing RA associated HLA-DRB1 alleles. CD4⁺ T cell clones from three normal individuals, NP-1, NP-2, and NP-3, were generated using the identical approach as described for the RA patients. TCR β chains of T cells with identical Vβ elements were sequenced and were found to be different (data not shown). Although a similar sample size of CD4⁺ T cell clones was analyzed as in the RA patients, only very few Vβ14⁺ and Vβ17⁺ CD4⁺ T cells were identified and could be sequenced. We, therefore, established T cell clones from presorted CD4⁺ Vβ17⁺ cells in two additional individuals, NP-4 and NP-5, and sequenced the TCR β chains from 25 and 28 Vβ17⁺ T cell clones, respectively. All TCR sequenced showed junctional diversity, suggesting that the clonal expansion within the circulating CD4⁺ T cell population is not a general phenomenon in normal individuals.

To address the hypothesis that the peripheral clonal expansion is related to the rheumatic disease, we used yet another approach to analyze the peripheral TCR repertoire in three additional normal individuals and three additional patients with early RA. CD4⁺ IL-2R⁻ T cells were separated from peripheral blood by cell sorting. The distribution of CDR3 sizes within one Vβ-Jβ combination was determined to screen for clonally expanded T cells within the CD4⁺ IL-2R⁻ fraction. In patients RA-1 and RA-2, clonotypes were only found within Vβ14⁺ and Vβ17⁺ T cells. Recent results by other groups have implicated Vβ3, Vβ14, and Vβ17 T cells in the pathogenesis of RA (13, 14, 27). We have therefore focused our analysis on these Vβ elements and have used the Vβ5.1 and Vβ8 elements as controls. The Jβ elements we have included in the analysis, Jβ1.1, Jβ1.2, Jβ1.5, Jβ2.1, Jβ2.3, Jβ2.5, and Jβ2.7, are the most frequent Jβ elements in the human repertoire and encompass ~70% of the T cell repertoire (Walser-Kuntz, D., C. Weyand, and J. Goronzy, manuscript in preparation). All V-J combinations in the three normal controls showed an unremarkable Gaussian distri-

Table III. Frequency of Clonotype RA-1-17A in Peripheral Blood and Synovial Fluid

	CD4 ⁺ Vβ17 ⁺ T cells		IL-2 responsive CD4 ⁺ Vβ17 ⁺ T cells	
	PBMC	Synovial fluid	PBMC	Synovial fluid
Month 7	30.5%	6.4%	8.9%	0.7%
Month 15	83%	85%	6%	15%

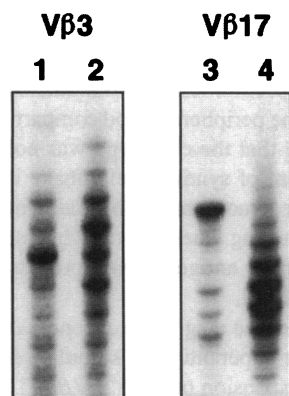


Figure 4. Identification of dominant clonotypes by V-J β specific amplification and CDR3 length analysis. CD4⁺ IL-2R⁻ T cells were purified from PBMC of a normal control (lanes 2 and 4) and patient RA-3 (lanes 1 and 3). cDNA was amplified by PCR with V β specific primers and a C β primer followed by amplification with the appropriate V β -J β specific primer set. Results are shown for the V β 3-J β 2.1 and V β 17-J β 2.3 sequences derived from peripheral CD4⁺ IL-2R⁻ T cells. Sequencing confirmed the dominant clonotypes in patient RA-3 (Table IV).

bution of TCRs expressing different CDR3 length (data not shown). Direct sequencing of selected V-J combinations confirmed that these Gaussian distributions did not contain an expanded clonal specificity which exceeded 25% of a particular V-J combination and could therefore be detected by direct sequencing. In contrast, deviations from the Gaussian distribution and dominance of one particular CDR3 length was seen for 12 V β -J β combinations in the three patients (Fig. 4). Sequencing of these 12 V-J combinations revealed dominant clonotypes in 7 out of the 12 samples sequenced. The TCR sequences are given in Table IV. Patient RA-3 had three, patient RA-4 three, and patient RA-5 one dominant clonotype. It is of interest to note that the T cell clones identified expressed a V β 3, V β 14, or V β 17 element.

Different clonotypes are independently regulated. The dominance of expanded clonotypes within V β 3⁺, V β 14⁺, and V β 17⁺ T cells suggested a common stimulatory mechanism in the different patients. However, the comparison of the CDR3 region did not allow any conclusion on the recognition of a shared conventional antigen. The identification of distinct clonotypes in the same patient provided the opportunity to compare the long-term time course of different T cell specificities and

thus to obtain indirect information on shared or distinct mechanisms driving the activation and clonal expansion of these clonotypes. T cell clonotype RA-1-17A was originally isolated from synovial fluid of patient RA-1 2 mo after disease onset. A second V β 17⁺ clonotype, RA-1-17C, was isolated from the peripheral blood 7 mo after disease onset. The two clonotypes utilized different J β elements, J β 1.2 and J β 2.7 (Table II). We monitored the frequencies of T cells expressing particular V β 17-J β combinations to address the question whether clonal expansion of distinct clonotypes occurred in parallel. The frequencies of T cells expressing the V β 17-J β 1.2 and the V β 17-J β 2.7 combinations were clearly distinct from the frequencies observed in normal controls (data not shown). Results for these two V-J combinations are shown in Table V. At the first time point, V β 17⁺ T cells expressing the J β 2.7 element were essentially undetectable in unselected CD4⁺ T cells but were detectable in IL-2 responsive CD4⁺ cells. At this time point, 65% of all V β 17⁺ transcripts used the J β 1.2 segment. The V β 17-J β 2.7 combination increased to 37% over the next 4 mo and returned to 16 and 9% in the following 8 mo. In contrast, the V β 17-J β 1.2 combination decreased to a frequency of 37% and then started to increase again. At the last time point analyzed, nearly the entire V β 17 compartment was constituted of T cells utilizing a J β 1.2 gene segment. Thus, monitoring of the V β 17-J β combination demonstrated major fluctuations in the repertoire of V β 17⁺ cells, suggesting that the extent of clonal expansion for two distinct clonotypes varied over time. Monitoring of the J β elements expressed by these clonotypes revealed that the changes in the frequencies were not synchronous, indicating that independent mechanisms were controlling the proliferation of these different clonotypes.

Discussion

This study identified an abnormality in the TCR repertoire of peripheral CD4⁺ T cells in patients with early RA. While analyzing the diversity of synovial fluid T cells, we found that several clonotypes in the CD4⁺ subset had undergone clonal expansion. Expanded clonotypes were not limited to the syno-

Table IV. Identification of Expanded CD4⁺ IL-2R⁻ T Cell Clonotypes in Peripheral Blood of Patients with Early RA by CDR3 Length Analysis and Direct Sequencing

		V β		N-D-N						J β							
RA-3	V β 3	S	S	R	G	Q	G	R	D	E	Q	F	F				
		AGC	AGT	CGC	GGA	CAG	GGA	CGG	GAT	GAG	CAG	TTC	TTC (2.1)				
	V β 14	S		R	E	G	Q	G	Y	P	E	A	F	F			
		AGC		AGA	GAA	GGA	CAG	GGG	TAC	CCT	GAA	GCT	TTC	TTT (1.1)			
V β 17	S	S	V	I	P	G	L	A	G	A	E	T	D	T	Q	Y	F
	AGT	AGT	GTA	ATT	CCG	GGA	CTA	GCG	GGC	GCG	GAG	ACA	GAT	ACG	CAG	TAT	TTT (2.3)
RA-4	V β 14	S	S	P	W	G	S					S	Y	E	Q	Y	F
		AGC	AGT	CCT	TGG	GGC	TCT					TCC	TAC	GAG	CAG	TAC	TTC (2.7)
	V β 17	S	S	T	P	G	R	A				N	T	E	A	F	F
		AGT	AGT	ACC	CCG	GGG	CGG	GCG				AAC	ACT	GAA	GCT	TTC	TTT (1.1)
V β 17	S	S	S	R	P	G	A	G				Y	G	Y	T	F	
	AGC	AGT	AGT	AGG	CCG	GGG	GCC	GGC				TAT	GGC	TAC	ACC	TTC (1.2)	
RA-5	V β 14	S	S	R	E	G	F					Y	G	Y	T	F	
		AGC	AGC	AGA	GAG	GGT	TTC					TAT	GGC	TAC	ACC	TTC (1.2)	

Table V. Frequencies of V β 17-J β Combinations in PBMC of Patient RA-1

Months after disease onset	Origin	V β 17-J β 1.2	V β 17-J β 2.7
2	CD4 ⁺ T cells	65%	< 1.0%
	IL-2R ⁺ CD4 ⁺ T cell	42%	5.4%
7	CD4 ⁺ T cells	37%	37.4%
	IL-2R ⁺ CD4 ⁺ T cell	16%	9.3%
11	CD4 ⁺ T cells	54%	15.8%
	IL-2R ⁺ CD4 ⁺ T cell	56%	5.6%
15	CD4 ⁺ T cells	90%	9.3%
	IL-2R ⁺ CD4 ⁺ T cell	33%	< 1.0%

vial compartment but rather represented dominant clonal populations within peripheral blood lymphocytes. The clonal dominance resulted in a significant restriction of the CD4⁺ T cell repertoire. We monitored clonal populations of CD4⁺ T cells over a study period of 1 yr. The frequencies of individual clonotypes fluctuated considerably and was not synchronously regulated for distinct clones. These data establish that the TCR repertoire of RA patients is majorly skewed due to the emergence of clonally expanded CD4⁺ T cells. The inability of RA patients to control clonal expansion in the CD4⁺ T cell pool may relate to an intrinsic defect in T cell generation and regulation. The systemic nature of the repertoire abnormality emphasizes that RA is a systemic disease in which the synovial membrane might only provide the microenvironment for a chronic inflammatory response.

Polyclonality of T cells has been considered a fundamental principle of the immune system. Only through the availability of an almost unlimited spectrum of TCR is the immune system equipped to recognize and eliminate the universe of antigens. The number of TCR specificities represented in the potential human T cell repertoire has been estimated to be at least 10¹⁵ (27). Thymic selection imposes some restriction on this diversity, however, at least 1% of T cells survive this selection process. Thus, identical TCR specificities in a sample of human T cells can only be encountered if the repertoire is severely skewed. Isolation of several copies of identical T cells requires substantial expansion of the precursor cell. Indeed, the identification of dominant sequences by direct sequencing and the quantification of clonotype specific sequences in CD4⁺ T cells demonstrated that the expanded clonotypes represented a significant proportion of CD4⁺ T cells sharing a particular V β -J β combination and that clonal expansion had led to a major skewing of the TCR repertoire.

Several mechanisms could cause the T cell repertoire abnormality seen in RA patients. Generally, T cells replicate after specific interaction of the TCR with the appropriate HLA-antigen complex. The extent of oligoclonal expansion reported here appears unusually high for an antigen specific response. Priming with antigen usually increases the frequency of circulating T cells specific for a complex antigen from ~ 1/100,000 to 1/5,000 T cells (28–30). The expanded clonotypes described here, however, were present at frequencies 0.1% to higher than 1% of all TCR molecules. Such high frequencies are generally only achieved for antigen specific responses in well defined

tissue compartments. The representation of identical TCR clonotypes in the peripheral blood and in the synovial fluid of the two RA patients could be the result of tissue infiltration of T cell specificities expanded in the periphery or the result of recirculation of in situ expanded specificities. The dominance of the clonally expanded T cells in the peripheral blood compartment of all patients and the finding that the clonotype was not enriched in the IL-2R⁺ compartment of synovial cells make it unlikely that T cells replicate in the synovium and migrate out of the joint. This raises the interesting question of whether expanded T cell clones result from an antigen-driven process and where the clonotypes are expanded.

In vivo TCR repertoire changes of the magnitude as reported here have only been observed after superantigenic stimulation (31). The preferential clonotypic expansion of V β 3⁺, V β 14⁺, and V β 17⁺ T cells is an interesting finding and would be compatible with a V β specific mechanism initiating T cell growth. These V β elements share a common structure in the HVR4 of the TCR β chain which has been implicated in superantigen binding (32, 33). Stimulation with a superantigen alone, however, does not result in the selection of dominant clonotypes within one V β element. In addition, we initially did not find a major skewing of the V β 3 and V β 17 frequencies when analyzed with anti-TCR mAb (data not shown). Only after one clonotype reached more than 80% of the V β 17 subset did we detect increased frequencies of V β 17⁺ CD4⁺ T cells in the blood. It is possible that the initial stimulus was provided through a superantigen followed by clonal selection and expansion. Experiments in murine models have suggested that exposure to a superantigen induces T cell proliferation followed by deletion of all T cell specificities except the ones which encounter antigen (34–36). This mechanism has been recently proposed to play a role in the pathogenesis of RA (13). In this model, we would have missed the time of superantigenic expansion, but would have captured the stage of antigenic selection in the patients described here. It is of particular interest to note that V β 3⁺, V β 14⁺, and V β 17⁺ T cell clones have been implicated by a number of investigators in synovial inflammation in RA (13, 14, 26, 37).

Clonal abnormalities of circulating T cells have been previously described in RA patients. These clonal expansions have been limited to CD8⁺ T cells. Generally, clonality among CD8⁺ T cells has been associated with Felty's syndrome (38, 39). In a subset of these patients, clonality in the CD8⁺ subset has been linked to increased numbers of large granular lymphocytes (38). Clonal expansion within the CD8 population has also been described in Felty's patients who do not present with an expansion of large granular lymphocytes (40). It has been proposed that such expanded clonotypes might exhibit suppressive or cytotoxic effector functions in the bone marrow resulting in a suppression of the granulopoiesis. In general, patients with Felty's syndrome have longstanding RA. Thus, the emergence of clonal CD8⁺ specificities may be a consequence of the chronic inflammatory response in these patients. It is, however, of interest to note that most of the patients with Felty's syndrome, as patients with other extra-articular rheumatoid organ manifestations, carry a double dose of disease associated HLA-DRB1 alleles (41). Three of the five patients described in this study also expressed two disease-associated HLA-DRB1 alleles and one of these patients, RA-1, has already

developed extra-articular manifestations in the early stages of disease.

DerSimonian et al. (42) have suggested that the clonal expansion of CD8 clonotypes may not be restricted to patients with Felty's syndrome but may be a more generalized feature of patients with RA. These authors have used mAbs to the V α 12 element to identify RA patients with largely increased numbers of V α 12⁺ CD8⁺ T cells in the peripheral blood. Sequence analysis confirmed the dominance of single clonotypes in these expanded T cell populations. Clonal expansion was found in ~ 10% of the RA population studied. None of the patients presenting with clonally expanded CD8⁺ T cells expressed the disease associated HLA-DR "shared epitope" on both haplotypes. Dominant clonotypes within the CD8 subset have also been identified in normal donors, although it appears to be a more prominent finding in RA (43, 44). There is emerging evidence that the repertoire of CD8⁺ T cells is smaller than expected and that clonal dominance, at least as a transient phenomenon, is not unusual. In contrast, clonal expansion within the CD4 subset appears to be a very unusual finding.

The expanded clonotypes described in this study expressed the CD4 marker and were negative for the CD8 marker. All of them were isolated from patients with very early disease. Thus, these CD4⁺ clonally expanded T cell populations are unlikely a result of chronic disease. Their presence during very early disease rather raises the possibility that they are involved in inducing disease. Dominant CD4⁺ T cell clonotypes are clearly not restricted to early disease but persist over time (Fig. 3) and can also be found in patients with established disease (unpublished observations). The independent fluctuation of two clonotypes further challenges considerations that clonal expansion results from T cell recognition of an arthritogenic antigen. Clone RA-1-17C fluctuated in a pattern resembling disease activity, whereas clone RA-1-17A did not (Table V). 7 mo into the disease, patient RA-1 had highly active disease with polyarthritis, anemia, and peripheral neuropathy documented by electromyography. At that time, the frequency of clone RA-1-17C peaked. Aggressive immunosuppressive therapy combining high doses of corticosteroids and methotrexate was initiated. Disease activity declined, and 8 mo later the patient had majorly improved but continued to have active disease. At that time, clone RA-1-17C was at minimal concentration, whereas clone RA-1-17A had reached a maximum of 83% of V β 17⁺ T cells. Correlation of disease manifestations with the concentration of different clonotypes in a series of patients might represent an approach to study their role in the disease.

In the current paradigm, the contribution of CD4⁺ T cells toward the inflammatory response characteristic for RA is understood as a local immune response in the synovial membrane. Here, we are describing a major abnormality in the composition of the total CD4⁺ T cell pool in RA patients. Distribution of oligoclonal T cell populations in the joint and in the peripheral blood were not suggestive of a synovial immune recognition as the site of T cell expansion. The data presented are compatible with a model suggesting that RA patients have a defect in T cell generation or regulation which leads to a majorly skewed TCR repertoire. Possible mechanisms include a different responsiveness toward stimulating and apoptosis inducing signals. By cytogenetic analysis, we have not been able to identify chromosomal abnormalities in expanded clonotypes (data not shown). We are proposing that regulation of T cell generation,

survival, and reactivity are altered in RA patients and that clonally expanded CD4⁺ T cells are an important component in the disease process. This model includes that RA is primarily a systemic disease and that synovitis is the result and not the cause of an altered composition of the TCR repertoire.

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